Experimental Eye Research 174 (2018) 59–69

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Ocular toxicity of reduced graphene oxide or graphene oxide exposure in mouse eyes

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ARTICLE INFO

Keywords:
Reduced graphene oxide
Graphene oxide
Ocular toxicity
In vivo
In vitro

ABSTRACT

With the wide application and mass production of nanoparticle products, environmental nanopollutants will become increasingly common. The eye is an important organ responsible for vision in most living organisms, and it is directly exposed to the atmosphere. Direct contact between the eye and nanoparticles in the environment can potentially lead to ocular damage. However, publications focusing on the eye-damaging potential of nanoparticles are scarce. Therefore, to evaluate the impact of nanoparticles on the eyes, we investigated the ocular toxicity of reduced graphene oxide (RGO) and graphene oxide (GO) using morphological and molecular biological methods *in vivo* and *in vitro* in the present work. The findings show that short-term repeated GO exposure can cause obvious intraocular inflammation, an incrassated corneal stromal layer, cell apoptosis in the cornea, iris neovascularization and significant cytotoxicity of rat corneal epithelial cells (rCECs), while RGO causes no significant ocular toxicity in mice.

1. Introduction

Recently, single sheets of graphene have been characterized as the thinnest two-dimensional material, with the basic structural unit of a benzene ring (Ferrari et al., 2006; Geim and Novoselov, 2009). Graphene oxide (GO), a derivative of graphene, has oxygen-containing groups, including hydroxyl groups, carboxyl groups and other groups (Gao, 2015; Stankovich et al., 2007). Similar in structure to pristine graphene, reduced graphene oxide (RGO), which contains residual oxygen and other heteroatoms, is traditionally prepared using GO produced with Hummers' method (Gensheimer Julia, 2014). RGO and GO are two typical carbon-based nanoparticles that are expected to become revolutionary materials in electronics and biomedical fields, which rely on their performance-optimizing properties. RGO and GO have greater application potential than other nanoparticles in several fields and have been widely produced and used for many purposes, including solar cells, aviation, biosensing (Kostarelos and Novoselov, 2014; Kumar et al., 2016; Xu et al., 2017), disease diagnosis (Chae et al., 2016; Wu et al., 2014), bacteriostasis (Akhavan and Ghaderi, 2010, 2012; Hu et al., 2010; Yan et al., 2011) and antiviral materials (Akhavan et al., 2012). Graphene-based nanoparticles are modified with substances such as polyethylene glycol, pectin and BSA. However, with their wide range of applications and mass production, these nanoparticles are also released into the environment through various avenues and cause potential environmental and biosafety hazards. Therefore, biosafety and environmental safety issues regarding these nanoparticles have been the subject of widespread public concern in recent years (Kovbasyuk and Mokhir, 2016; Patlolla et al., 2016; Pecoraro et al., 2018; Wen et al., 2015; Zhang et al., 2017). To address these concerns, an efficient proactive risk assessment must be conducted before using these nanomaterials.

In recent years, considerable re (Xu et al., 2017)search on nanotoxicity has provided references for correlational studies. Some studies indicate that graphene-based nanoparticles can enter the bloodstream and translocate to other organs and can also circulate in the nervous system (Akhavan, 2016; Lee et al., 2011); carbon-based nanoparticles taken orally or intravenously injected into the body are mainly distributed in the liver, kidneys and lungs and can lead to inflammation in these organs (Duch et al., 2011; Kovbasyuk and Mokhir, 2016; Patlolla et al., 2016; Qi et al., 2017; Seabra et al., 2014; Yang et al., 2013). The toxicity associated with these nanoparticles varies with their size and structure (Gurunathan et al., 2013; Seabra et al., 2014). Many studies evaluating the cytotoxicity of carbon-based nanoparticles have been published; for example, nanoparticle-induced apoptosis, cell death and inflammation have recently been reported (Chowdhury et al., 2013; Lv et al., 2012; Sasidharan et al., 2012; Singh et al., 2012; Vallabani et al.,

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https://doi.org/10.1016/j.exer.2018.05.024
Received 3 February 2018; Received in revised form 14 April 2018; Accepted 23 May 2018
Available online 24 May 2018
0014-4835/

2011; Yue et al., 2012; Zhang et al., 2010). Although studies have reported that phospholipids and statins can effectively relieve toxicities caused by nanoparticles (An et al., 2017; Qi et al., 2017), the biological and environmental security of carbon-based nanomaterials remains unclear. Therefore, environmental and occupational deposition onto superficial organs, such as the eye and skin, may be major routes of graphene and graphene derivative exposure among humans and other organisms (Wu et al., 2016).

The eye is an important organ with a complex optical system in most animals, and it is considered the prototypical immune-privileged tissue (Benhar et al., 2012). Although it has several protective mechanisms, the eye is vulnerable to external influences such as ultraviolet radiation, microbial infection, dust and external invaders due to its direct contact with the environment. The wide application of nanoparticles requires awareness of ocular vulnerability and the potential for irreversible sight-threatening consequences.

The exact mechanism of ocular toxicity due to graphene-based materials is complex and has yet to be identified. Studies in the literature reporting primary irritation tests for nanoparticles in the eye are limited. These studies mainly investigated the cytotoxicity of GO on human retinal pigment epithelium cells, corneal epithelium cells and conjunctiva epithelium cells, and test of acute eye irritation were performed in rabbits (Wu et al., 2016; Yan et al., 2012). However, due to differences in experimental models/animals, which significantly affect study results, different outcomes were observed among these studies. Therefore, investigating the ocular toxicity of nanoparticles and filling this gap in the research is important.

In this work, RGO and GO were selected as test materials, and we focused on their ocular toxicities *in vivo* and *in vitro*. To the best of our knowledge, this is the first detailed study of this kind to investigate the ocular toxicities of RGO and GO. This research can provide basic data to help reveal the harmful health effects of RGO and GO nanomaterials.

2. Materials & methods

2.1. Preparation and characterization of the tested materials

Natural graphite was purchased from Suzhou Heng Qiu Technology Co. Ltd. (Suzhou, China). GO was obtained according to Hummers' method (Jr and Offeman, 1958). Briefly, natural graphite was oxidized into GO with H_2SO_4 and $KMnO_4$ (3 g: 400 mL: 18 g). The mixture was stirred for three days to ensure complete oxidation. The oxidation reaction was terminated by the addition of H_2O_2 when the entire mixture turned yellow. The yellow substance was washed several times with 1.0 M aqueous HCl solution and deionized water until a pH of 4–5 was achieved. The oxidized graphene was dispersed via ultrasonication for 2 h, followed by centrifugation to discard unoxidized graphite. Finally, the sample was freeze-dried to obtain GO in solid form.

According to the method of Park (Park et al., 2011), 100 mg GO was mixed with 100 mL water to yield an inhomogeneous yellow-brown dispersion. This dispersion was sonicated until it became clear and had no visible particulate matter. Then, 100 mL of 32.1 mmol hydrazine hydrate was added, the solution was heated in an oil bath at 100 °C, and then the mixture was placed under a water-cooled condenser for more than 24 h, resulting in gradual precipitation of the reduced GO (RGO) as a black solid. This product was isolated and washed with 100 mL water 5 times and with 100 mL methanol 5 times. Finally, the product was dried at 45 °C in a vacuum oven, and the RGO was obtained.

The solid RGO and GO were confected to a suspension of 10 μg/μL via ultrasonication for 40 min. The obtained samples of RGO and GO were used for further testing. Transmission electron microscopy (TEM), Raman shift, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) were used for morphological characterization and structural analyses of lamellar GO and RGO.

2.2. Toxicity assessment in vivo

2.2.1. Effective doses of RGO and GO

Eight-week-old Kunming mice (female) with a normal weight of 15–18 g were provided by the Laboratory Animal Center of the Medical College, Lanzhou University, Gansu, China. All animals were maintained in a controlled environment at 21 °C–22 °C with lights on from 08:00 to 20:00 h. The mice were allowed free access to food and tap water. The animals were raised and used humanely according to the Principles of Laboratory Animal Care Formulated by the National Society of Medical Research and the guidelines of the US National Institutes of Health. The mice were grouped randomly (5 mice/group) before the experiment, and the right conjunctival sacs of the mice in the experimental groups were exposed to 10-μL doses of the RGO and GO suspensions (25, 50 and 100 μg/mL); the left eyes were not exposed to these materials. The sham-operated group was exposed to double-distilled water. All mice were exposed to the RGO and GO suspensions once per day for a total of 7 d. Before exposure, all mice were narcotized with 50 μL of 5% chloral hydrate. Based on the results of the above experiment, the 50 μg/mL concentration was selected as the test dose in the following experiments.

2.2.2. Histopathology assay

The eyeballs of the treatment and control group mice were obtained via standard surgery. Tissues were stored in 10% buffered formalin and processed for routine histology with HE staining. Paraffin sectioning was used to obtain 5-μm-thick sections. The tissues were microscopically observed using a microscopy imaging system (AxioScope.A1, Oberkochen, Germany).

2.2.3. Corneal cell apoptosis assay in vivo

The histology sections were prepared, and an *in situ* fluorescein TUNEL cell apoptosis detection kit (Transgene FA201-02, China) and Hoechst 33258 (0.5 μg/mL) were used to detect the apoptosis of corneal cells. The apoptotic cells were labeled with cyan, and the labeled cells were microscopically observed using a fluorescence microscope system (Leica SP8, Solms, Germany).

2.2.4. Iris angiography

Approximately 12 μL/day of 50-μg/mL RGO and GO solutions were applied directly to the right eye of the mice for 7 d as the model group, and all mice were injected with labeling solution (mixed with 10 mL of 10% formalin, 10 mL of 30%, 5 mL of 2% direct blue 53, 50 μL of 10% Ammonium Persulfate (APS), and 10 μL of Tetramethylethylenediamine (TEMED)) using a heart perfusion technique and maintained at 37 °C for 1 h. The eyeballs were obtained, and the irises were separated under an anatomical lens (ZEISS Stemi DV4) and observed using a fluorescence microscope system (Leica SP8, Solms, Germany).

2.2.5. Toxic mechanism in vivo

A total of 12 μL/day of 50-μg/mL RGO and GO solutions was applied directly to the right eyes of the mice for 7 d as the model group; sham-operated mice were regarded as the control group. The right eyes of the model mice and control mice were collected for the following experiments.

For total protein extraction, fresh eyeballs were retrieved, weighed and placed in tissue lysis buffer [30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS (W/V), pH 7.5] in a proportion of 4 μL of buffer/mg of tissue. After ultrasonication at a low temperature, the mixture was centrifuged at 14,000 g for 15 min at 4 °C to collect total proteins in a solution that was stored at −80 °C. For western blotting, the total proteins were denatured fully in a boiling water bath for 5 min. The denatured proteins were separated in 12% SDS-PAGE under 100 V for 90 min and transferred onto a polyvinylidene fluoride (PVDF) membrane at 300 mA to process the routine western blots. The PVDF membrane was washed with washing buffer (Tris-buffered saline with